STIC-ILL

From: Vogel, Nancy
Sent: Tuesday, September 16, 2003 3:50 PM
To: STIC-ILL
Subject: refs. for 09/744488

please send me the following:

Holtmaat et al., (1996) Brain. Res. Mol. Brain Res. 41, 148-156).

53. Miller, D. S., P. Safer and L. K. Miller, 1986, An insect baculovirus host-vector system for high level expression of foreign genes, In Genenic Engineering, Vol. 8, Setlow, J. K. and Hollaender, A,. eds. Plenum Publishing Corp., New York, pp. 277-298.

Proc. Natl. Acad. Sci. 92:10099-10103 (1995)

Proc. Natl. Acad. Sci. 93:2348-2352 (1996)

Human Gene therapy 7:1937-1945 (1996)

Human Gene Therapy 8:2011-2018 (1997)

EXP NEUROL, (1990) 110 (1), 11-24.
J BIOL CHEM, (1992) 267 (17), 12275-12283.
Journal of Neurochemistry, (1994) Vol. 62, No. 2, pp. 471-478.
Journal of Biological Chemistry, (1994) Vol. 269, No. 1, pp. 755-759.
Journal of Neurochemistry, (1994) Vol. 62, No. 3, pp. 825-833.
Journal of Neurochemistry, (1994) Vol. 62, No. 3, pp. 825-833.
Journal of Biological Chemistry, (1996) Vol. 271, No. 48, pp. 30986-30991.
Biochemical Journal, (1997) Vol. 324, No. 2, pp. 461-466.
Biochemical and Biophysical Research Communications, (October 22, 2000) Vol. 277, No. 2, pp. 355-360.
Growth Factors, (1990) 3/1 (1-13).
Growth Factors, (1991) 5/2 (115-127).
Biochemical Journal, (1997) Vol. 326, No. 2, pp. 407-413.
Virology, (1995) Vol. 208, No. 1, pp. 328-335.
Nucleic Acids Research, (1997) Vol. 25, No. 9, pp. 1864-1865.
PROC NATL ACAD SCI U S A, (1992) 89 (9), 4094-4098
.Protein Engineering, (1994) Vol. 7, No. 4, pp. 537-541

thanks,
Nancy Vogel
Art Unit 1636
CM12 C01
(703) 308-4548

Journal of Neurochemistry
Raven Press, Ltd., New York

BDNF

Production and Characterization of Recombinant Rat Brain-Derived Neurotrophic Factor and Neurotrophin-3 from Insect Cells

Alessandro Negro, Adriana Tavella, *Claudio Grandi, and Stephen D. Skaper

*Fidia Research Laboratories, Fidia S.p.A., Abano Terme, and *Department of Organic Chemistry, University of Padua, Italy*

Abstract: Rat brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were engineered for expression in a baculovirus-infected *Spodoptera frugiperda* insect cell system. The BDNF and NT-3 from the culture supernatants were purified by ion-exchange and reverse-phase chromatography to apparent homogeneity. The purification procedure yielded ~2 mg of pure rat BDNF or NT-3 per liter of culture supernatant. A single N-terminus only was found for either secreted molecule and was analogous to that predicted from the corresponding cDNA sequence. The recombinant neurotrophins obtained were also homogeneous with regard to molecular weight and amino acid sequence. In their native conformation, the insect cell-produced rat BDNF and NT-3 molecules were homodimers consisting of 119 amino acid polypeptide chains. Thus, although the genes transfected into the *S. frugiperda* cells coded for proBDNF or proNT-3, the BDNF and NT-3 recovered after purification were >95% fully processed, mature protein. Mature recombinant rat BDNF and NT-3 were found not to be significantly glycosylated. Pure, recombinant rat BDNF and NT-3 promoted the survival of embryonic dorsal root ganglion neurons in the low picomolar range. Because recombinant rat BDNF and NT-3 can be obtained in large quantities, purified to near homogeneity, and are identical in amino acid sequence to the corresponding human proteins, they are suitable for evaluation in animal models. **Key Words:** Brain-derived neurotrophic factor—Neurotrophin-3—Baculovirus—Purification—Rat.
J. Neurochem. **62,** 471–478 (1994).

The development and maintenance of the vertebrate nervous system depends on neuronal survival proteins known as neurotrophic factors (Snider and Johnson, 1989). Nerve growth factor (NGF) was the first neurotrophic molecule demonstrated to be required for normal development, particularly in the PNS (Levi-Montalcini, 1987). That NGF maintains the survival of only certain neuronal subtypes suggests that there exist additional molecules with distinct specificities that fulfill the role of target-derived, retrogradely transported neurotrophic factors. Indeed, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3; also described as hippocampus-derived neurotrophic factor) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and neurotrophin-4/neurotrophin-5 (Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992) have recently been identified as members of an NGF-related family of gene products now termed neurotrophins.

These neurotrophic factors are synthesized as precursors and upon proteolytic cleavage, the mature, biologically active forms are generated. Mature BDNF and NT-3 are 119 amino acid residue polypeptides, with NGF being shorter by one amino acid residue. At physiologically relevant concentrations, NGF (Bothwell and Shooter, 1977) as well as BDNF and NT-3 (Radziejewski et al., 1992) exist as tightly associated dimers. The neurotrophins share ~55% sequence identity including six conserved cysteine residues that, in NGF (Angeletti and Bradshaw, 1971), form three intramolecular disulfide bridges. Despite their similarity in primary structure, BDNF and NT-3 display neuronal specificities different from those of NGF (Thoenen, 1991).

Neurotrophic factors are found in only minute levels in their respective target tissues and are consequently very difficult to purify, making high-level expression of the protein using recombinant DNA technology a desirable alternative. Small amounts of biologically active BDNF (Leibrock et al., 1989) or NT-3 (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) have been

Received March 30, 1993; revised manuscript received June 15, 1993; accepted June 22, 1993.

Address correspondence and reprint requests to Dr. A. Negro at Fidia Research Laboratories, Via Ponte della Fabbrica 3/A, 35031 Abano Terme, Italy.

Abbreviations used: BDNF, brain-derived neurotrophic factor; CD, circular dichroism; NGF, nerve growth factor; NT-3, neurotrophin-3; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sf-9, *Spodoptera frugiperda*; TFA, trifluoroacetic acid.

obtained from transiently transfected mammalian cell lines or bacterial expression systems (Negro et al., 1992), with somewhat greater yields having been described using stably transfected Chinese hamster ovary cells for BDNF (Rosenthal et al., 1991) or a vaccinia virus expression vector for NT-3 (Götz et al., 1992). However, no source for milligram quantities of properly folded, biologically active BDNF or NT-3 has been reported to date. We have now produced the recombinant proteins using baculovirus-infected *Spodoptera frugiperda* (Sf-9) insect cells. This expression system has already been used successfully with NGF (Barnett et al., 1990, 1991; Buxser et al., 1991). It yields more protein than other procedures and is less time-consuming than the generation of stable cell lines expressing foreign proteins. This report describes the expression, isolation, and physicochemical characterization of recombinant rat BDNF and NT-3.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories and used according to the supplier's directions. Complementary DNA was prepared using a DNA kit (Stratagene). Deoxyoligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer. DNA isolation, transformation, plasmid preparation, and other basic procedures were performed according to techniques previously described by Sambrook et al. (1989). Plasmids were constructed in *E. coli* HB101 and the sequences verified by double-stranded sequencing (Sanger et al., 1977). Sf-9 cells were obtained from the American Type Culture Collection (GRL1711) and were maintained in TNM-FH medium (Summers and Smith, 1987). TNM-FH medium contains Grace's medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum, 0.3% lactalbumin hydrolysate, 0.3% yeastolate, and gentamicin (50 μg/ml). Sf-9 cells were propagated as monolayer cultures at 28°C.

Construction of the expression plasmid for BDNF

The rat BDNF gene cloned in the eukaryotic expression vector pSVT7 (Negro et al., 1992) was cut at *Xba*I and *Bam*HI and cloned in the transfer vector pVL1392 (Invitrogen) between the same restriction sites. The plasmid thus obtained was termed pVLBDNF (Fig. 1).

Construction of the expression plasmid for NT-3

Two oligonucleotides derived from the 5′ and 3′ coding regions of the rat NT-3 gene (Maisonpierre et al., 1990) were used as primers for amplification of the complete coding region by polymerase chain reaction (Saiki et al., 1988), using rat brain complementary DNA as template. Restriction sites *Xba*I and *Sal*I were incorporated into the primers. The sequences of the deoxyoligonucleotide primers were: forward: 5′ TTTCTAGATGTCCATCTTGTTTTATGTGA 3′ and reverse: 5′ AAGTCGACTATGTTCTTCCGATTTTTCTTGAC 3′. The amplified sequences were cut at *Xba*I and *Sal*I and cloned first into pGEM-3 (Promega), and then into pSVT7 at the same restriction sites, and the resulting sequences verified for correctness (Sanger et al., 1977). The rat NT-3 gene cloned in pSVT7 was cut at *Xba*I and *Bam*HI and cloned in pVL1392, as for BDNF. The plasmid thus obtained was termed pVLNT-3.

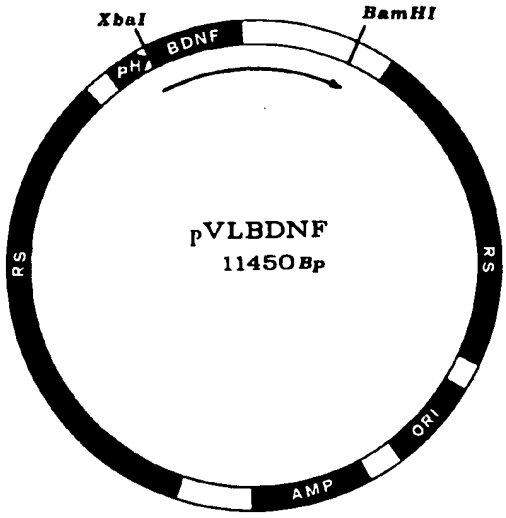

FIG. 1. Structure of the expression transfer vector for rat BDNF (analogous for NT-3). DNA fragments encoding the rat BDNF (or NT-3) precursor protein were generated by polymerase chain reaction and a rat brain cDNA as template. The polymerase chain reaction products obtained were cloned in the pSVT7 plasmid. The resulting plasmids were cut at *Xba*I and *Bam*HI and cloned in pVL1292 between the *Xba*I and *Bgl*II restriction sites. Arrow indicates the direction of transcription, the black box the mature (BDNF) protein. RS, recombination sequences; ORI, origin bacterial replication; AMP, ampicillin resistance site; pH, polyhedrin promoter.

Recombinant baculovirus was produced using the Baculogold transfection kit (Pharmigen). In brief, Sf-9 in 60-mm culture dishes was cotransfected with 2 μg of plasmid pVLBDNF or pVLNT-3 and 0.5 μg of linearized baculovirus. The cells were incubated in transfection buffer (Pharmigen) at 27°C for 4 h, and then shifted to TNM-FH medium supplemented with 10% fetal calf serum. After 4 days the culture supernatant was collected and assayed for biological activity. Part of this supernatant was used for further infection and virus amplification.

Large-scale production and purification of recombinant BDNF and NT-3

Five hundred milliliters of a suspension of Sf-9 cells (2×10^6 cells/ml) in Grace's medium supplemented with 5% fetal calf serum was infected with 20 ml of culture supernatant (4×10^8 plaque-forming units/ml). After 80 h at 28°C the culture medium was collected by centrifugation (12,000 *g*, 20 min), filtered through a 0.45-μm filter (Millipore), and directly applied to a column (2 × 10 cm) of S-Sepharose (Pharmacia) equilibrated with 150 m*M* NaCl/10 m*M* sodium phosphate, pH 6.8. After washing with 150 m*M* NaCl/10 m*M* sodium phosphate (pH 7.4), the recombinant protein was eluted with increasing NaCl concentration (150–600 m*M*) in 20 m*M* bicine, pH 8.5. Fractions containing biological activity were pooled, concentrated using

an Amicon concentrator (YM10 membrane), and then applied to a reverse-phase C8 HPLC column (4.6 × 150 mm) (Vydac) equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted using a gradient of 20–60% acetonitrile in 0.1% TFA over 60 min (0.5 ml/min). The eluted protein was dried under vacuum (Speed-Vac) and reconstituted in 100 m*M* NaCl/50 m*M* sodium acetate, pH 5.0.

Analytical methods

Protein concentrations were estimated by use of a dye binding assay (Bio-Rad). Amino acid analyses were performed using a Beckman 6300 amino acid analyzer. Automated Edman degradation of purified samples was performed on an Applied Biosystems 477A protein sequencer. Phenylthiohydantoin-derivatized amino acid peaks were identified on line.

Dimer/monomer equilibrium analysis

The monomeric and dimeric forms of BDNF and NT-3 were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. Purified recombinant proteins were added to electrophoresis sample buffer (50 m*M* Tris-HCl, 0.1% SDS, pH 8.0). The samples were loaded directly onto 9–22% gradient gels and electrophoresed (Phast System, Pharmacia). Proteins were visualized by silver staining and the relative abundance of proteins determined with a laser densitometer (Camag).

The molecular weights of BDNF and NT-3 were also analyzed by molecular sieve chromatography on a Superdex 75 (0.3 × 32 cm) column with a Smart System (Pharmacia) in 20 m*M* sodium phosphate buffer, pH 7.4. The elution profile was monitored by recording the absorbance at 280 nm. The apparent molecular weights of BDNF and NT-3 were interpolated using a calibration curve obtained from the elution volumes of the following standard proteins: cytochrome *c*, 12.3 kDa; myoglobin, 17 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; phosphorylase *b*, 94 kDa.

Circular dichroism (CD)

CD spectroscopy was performed between 200 and 250 nm on a Jasco J-500A spectropolarimeter, using 0.1-cm quartz cuvettes. The spectra were recorded at 25°C in 10 m*M* phosphate buffer, pH 7.4. A protein concentration of 0.15 mg/ml was used. Measurements were taken at 1-nm intervals with a 5-s constant and were averaged over 10 scans. Ellipticity was calculated using the following equation: $(\Theta) = \Theta \cdot PM/b \cdot c \cdot 10$ where Θ represents the instrumental ellipticity, PM is the molecular weight for an "average" amino acid residue (114.1K in the case of BDNF and 114.4K in the case of NT-3), b is the path length of the cuvette (cm), and c is the concentration of protein (mg/ml).

Carbohydrate analysis

Fifty micrograms of the respective recombinant protein was treated with 2 *M* HCl for 2 and 4 h at 100°C, as described by Hardy et al. (1988). Dried samples were derivatized with dansylhydrazine, and the dansylated sugars were resolved over a reverse-phase HPLC C18 column (Mopper and Johnson, 1983).

Assay for neurotrophic activity

Biological assays were performed using dissociated sensory neurons prepared from dorsal root ganglia of 9-day-old chicken embryos, as described by Skaper et al. (1990). The ganglionic cell suspension was preplated to reduce the number of nonneuronal cells and yielded cultures containing >95% neurons. Neurons were plated onto laminin/polyornithine-coated plastic, 6-mm-diameter microwells (96-well microtest plate, Falcon) and surviving neurons counted after 48 h.

RESULTS AND DISCUSSION

Production of BDNF and NT-3

Expression of NGF in insect cells infected with a recombinant baculovirus vector containing the human NGF gene has previously been described (Barnett et al., 1990, 1991). We have now prepared expression transfer vectors for rat BDNF and NT-3 for infection of Sf-9 cells. The recombinant viruses were obtained using the Baculogold system with a linearized virus, which allows achieving a homologous recombination while bypassing the time-consuming work of plaque purification (Summers and Smith, 1987). By the use of polymerase chain reaction it was possible to position the genes for BDNF and NT-3 in proximity to the regulatory sequences (the polyhedrin promoter). The untranslated 5′ and 3′ regions were removed from the DNA constructs to avoid possible deleterious effects on mRNA stability, as is the case for some cytokines (Shaw and Kamen, 1986). Splicing and polyadenylation sites are provided by SV40. Taken together, these procedures yielded comparable expression transfer vectors for the two neurotrophins.

The conditioned medium harvested from infected Sf-9 cells contained neurotrophic activity that allowed the survival of embryonic sensory neurons; when a serial dilution of the medium was assayed for biological activity, half-maximal activity was obtained at a dilution factor of ~10,000-fold. Medium from mock-infected Sf-9 cells was not active. This result showed that the recombinant BDNF and NT-3 proteins were expressed at high levels and constituted a significant proportion of the total protein exported into the medium. Relatively few plaques were obtained after the first cotransfection, although all expressed one or the other neurotrophin. Amplification of the recombinant virus at least three times was necessary to achieve high-titer synthesis.

Protein purification

Eighty hours after infection, culture medium was collected by centrifugation, filtered, and loaded onto an S-Sepharose fast-flow, cation-exchange column (see Materials and Methods). On development of the column with a sodium chloride gradient, NT-3 eluted with 0.3 *M* NaCl at pH 8.5 (Fig. 2); BDNF displayed a similar chromatographic behavior (not shown). Fractions containing biological activity were pooled, concentrated, and purified further using a Vydac C8 reverse-phase HPLC column. An acetonitrile gradient was applied and the NT-3 eluted at 30% acetonitrile

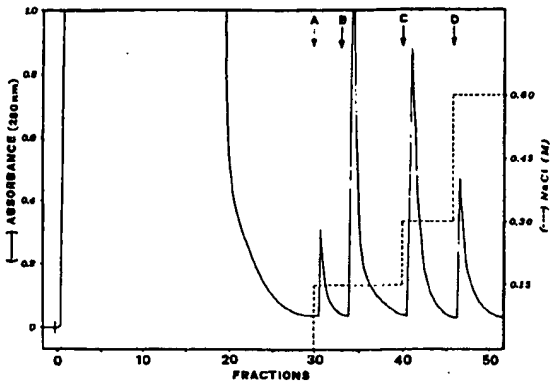

FIG. 2. S-Sepharose chromatography of recombinant rat NT-3. Culture fluid from insect cells infected with the recombinant baculovirus vector was loaded onto an S-Sepharose cation-exchange column (2 × 10 cm) with 150 m*M* NaCl, 10 m*M* sodium phosphate (pH 6.8). Retained proteins were eluted with a stepwise NaCl gradient (dashed lines). A, 150 m*M* NaCl, 10 m*M* sodium phosphate (pH 7.4); B, 150 m*M* NaCl, 20 m*M* bicine (pH 8.5); C, 300 m*M* NaCl, 20 m*M* bicine (pH 8.5); D, 600 m*M* NaCl, 20 m*M* bicine (pH 8.5). Elution of proteins was monitored by absorbance at 280 nm (solid lines). Fractions (40–45) containing NT-3 were identified by bioassay and pooled for further purification.

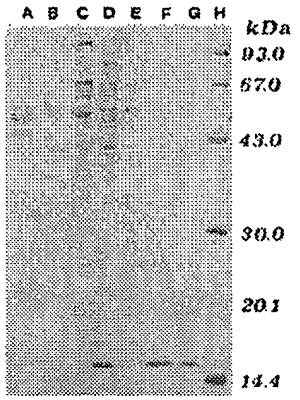

FIG. 4. Recombinant rat BDNF and NT-3 analyzed by SDS-PAGE. Samples were run under reducing conditions (5% β-mercaptoethanol) in 12% SDS-PAGE. Staining with Coomassie Brilliant Blue revealed that both preparations were at least 98% pure; the purified recombinant proteins had the electrophoretic mobility expected from their deduced molecular masses. Lanes A–E, pooled eluate fractions from S-Sepharose column loaded with culture fluid from Sf-9 cells infected with pVLBDNF: A, 150 m*M* NaCl, pH 6.8; B, 150 m*M* NaCl, pH 7.4; C, 150 m*M* NaCl, pH 8.5; D, 300 m*M* NaCl, pH 8.5; E, 600 m*M* NaCl, pH 8.5. Lane F, BDNF after reverse-phase HPLC. Lane G, NT-3 after reverse-phase HPLC. Lane H, molecular mass standards (kDa).

(Fig. 3). Recombinant BDNF eluted slightly later than did NT-3 in this system (data not shown). Fractions judged to contain >98% pure product by SDS-PAGE were pooled and lyophilized to remove organic solvents. Recovery of BDNF or NT-3 in these fractions was typically 50% of the amounts loaded. Approximately 2 mg of pure rat NT-3 or BDNF was recovered from each liter of supernatant. Although the expression transfer vectors for BDNF and NT-3 are substantially equivalent, infected Sf-9 cells consistently produced more NT-3 than BDNF (data not shown), perhaps due to different degrees of processing of the respective prepro sequence, as found for neurotrophin-5 (Berkemeier et al., 1991). The final purity of the recombinant proteins was confirmed by stained SDS-PAGE gels (Fig. 4). Under reducing conditions the recombinant proteins had the electrophoretic mobility of an ~15-kDa species, consistent with deduced monomeric molecular masses of 13,582 Da and 13,653 Da for BDNF and NT-3, respectively. The slighter greater apparent molecular weight under reducing conditions probably reflects the unfolded nature of the protein; similar molecular weights for BDNF and NT-3 on reducing SDS-PAGE gels have been reported by others (Rosenthal et al., 1990, 1991; Götz et al., 1992). No second band in the BDNF lane below the band at 15 kDa was seen, in contrast to human BDNF expressed in Chinese hamster ovary cells (Rosenthal et al., 1991).

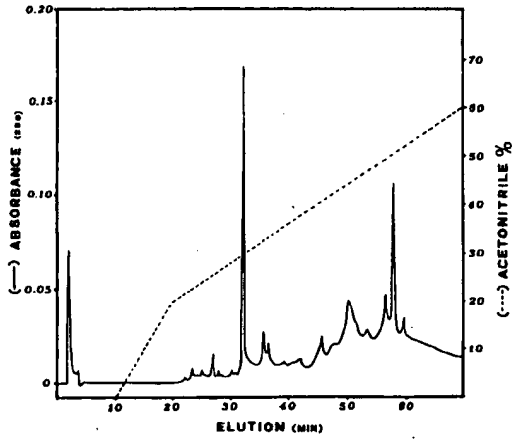

FIG. 3. Reverse-phase HPLC purification of recombinant rat NT-3. Pooled concentrated fractions from the S-Sepharose column (Fig. 2) were applied to a Vydac C8 reverse-phase HPLC column (0.46 × 150 mm) equilibrated in aqueous 0.1% TFA. Retained proteins were eluted with an acetonitrile gradient in 0.1% TFA (dashed line). Eluted proteins were monitored by absorbance at 220 nm (solid lines). Fractions containing NT-3 were identified by biological activity. The fraction eluting at 33 min contained at least 98% pure NT-3 as judged by silver-stained PAGE gels.

Neurotrophic activity

The ED_{50} of purified recombinant rat BDNF and NT-3 on chicken embryonic dorsal root ganglion neurons (see Materials and Methods) was determined to be ~0.4 ng/ml for either protein, i.e., close to the dissociation constants for high-affinity neurotrophin

TABLE 1. *Survival of cultured chicken sensory neurons*

Culture addition	Surviving neurons per well
NGF	1,195 ± 161
BDNF	957 ± 56
BDNF + NGF	1,759 ± 148[a]
NT-3	545 ± 57
NT-3 + NGF	1,384 ± 69

Embryonic day 9 dorsal root ganglion neurons were plated (2,000 per well), incubated for 48 h, and counted. Values are the mean of triplicate determinations ± SD. NGF (50 ng/ml), BDNF (25 ng/ml), and NT-3 (25 ng/ml) were used at concentrations at which maximal neuronal survival is observed with any of the three factors. NGF was purified from mouse submaxillary gland (Mobley et al., 1976).

[a] $p < 0.05$ vs. NGF alone (one-way ANOVA followed by Duncan's post hoc test).

receptors (Sutter et al., 1979; Rodriguez-Tébar and Barde, 1988). Neuronal survival with maximal concentrations of purified BDNF and NT-3 corresponded to 48 and 27% of the neurons plated (Table 1). Thus, NT-3 supports the survival of fewer sensory neurons than either BDNF or mouse submaxillary gland NGF (60%) under the same experimental conditions. Similar degrees of cell survival with recombinant BDNF and NT-3 in this system have been described by others (Leibrock et al., 1989; Rosenthal et al., 1990; Götz et al., 1992). Furthermore, the survival activity of BDNF on cultured dorsal root ganglion cells was roughly additive with that of NGF (Table 1). There was a trend for NGF to be additive also with NT-3, although the values did not reach statistical significance. Such additive effects of NGF and BDNF on the survival of dissociated sensory ganglion neurons are in agreement with earlier reports (Leibrock et al., 1989; Suter et al., 1992). These data clearly demonstrate the biological uniqueness of the two obtained recombinant proteins, both from each other and from NGF.

N-terminal amino acid sequence

N-terminal sequencing for 15 cycles yielded the following sequences: HSDPARRGELSVXDS for rat BDNF and YAEHKSHRGEYSVXD for NT-3. These sequences are identical to the respective DNA-deduced sequences (Leibrock et al., 1989; Hohn et al., 1990). The expected N-terminus was found for each neurotrophin, indicating that in insect cells proBDNF and proNT-3 undergo correct proteolytic processing to the mature polypeptide. Furthermore, the amino acid compositions of the recombinant BDNF and NT-3 were in good agreement with those predicted from the corresponding DNA sequences (Table 2), showing that no additional extensive N-terminal or C-terminal processing takes place. The precise removal of prepro sequences for the neurotrophins thus does not appear to be class-specific (Rosenthal et al., 1991; Götz et al., 1992), although prepro BDNF produced in *E. coli* is not processed (Negro et al., 1992).

Glyc sylation

Insect cells can both *N*- and *O*-glycosylate heterologous mammalian proteins (Luckow and Summers, 1988). In addition, insect cells are capable of trimming high mannose residues (Jarvis and Summers, 1989) and of forming complex carbohydrates from the high mannose structures (Davidson et al., 1990). Mature β-NGF contains a potential glycosylation site (Ullrich et al., 1983; Murphy et al., 1989); however, mature human NGF produced in insect cells is not significantly glycosylated (Barnett et al., 1991). Although neither mature NT-3 (Rosenthal et al., 1990) nor mature BDNF (Leibrock et al., 1989) contains consensus sequences for *N*-glycosylation, it was of interest to determine whether recombinant rat BDNF and/or NT-3 produced in insect cells is glycosylated. When subjected to SDS-PAGE analysis, BDNF and NT-3 from Sf-9 cells migrated to the same position as did mature rat BDNF from *E. coli* (Negro et al., 1992), the latter not being glycosylated, as bacteria lack the requisite enzymes. A sensitive chemical analysis was used to examine possible glycosylation of recombinant BDNF and NT-3. The BDNF and NT-3 samples were subjected to hydrolysis with HCl (for amino sugar determination) or TFA (for neutral sugar determination). The released monosaccharides were identified by reverse-phase HPLC (see Materials and Methods). Only trace amounts of mannose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine were detected (data not shown), indicating a lack of signifi-

TABLE 2. *Amino acid analysis of recombinant rat NT-3 and BDNF*

	Amino acid composition			
	NT-3		BDNF	
Amino acid	Expected	Found	Expected	Found
Asx	11	11.3	9	9.3
Glx	11	11.5	9	9.7
Thr	9	8.5	12	10.8
Ser	12	11.4	10	9.4
Pro	2	1.7	3	3.2
Gly	8	8.6	9	9.5
Ala	5	5.2	5	5.4
Cys	6	ND	6	ND
Val	9	9.6	9	9.2
Met	0	0.3	3	2.5
Ile	7	7.3	6	6.5
Leu	5	5.1	5	5.5
Tyr	5	5.3	4	4.1
Phe	1	1.3	2	2.5
Lys	10	9.7	11	10.5
His	4	3.2	2	1.5
Arg	10	9.3	11	10.6
Trp	4	ND	3	ND

Theoretical values are derived from the DNA sequence (Leibrock et al., 1989; Hohn et al., 1990). Tryptophan and cysteine contents were not determined (ND).

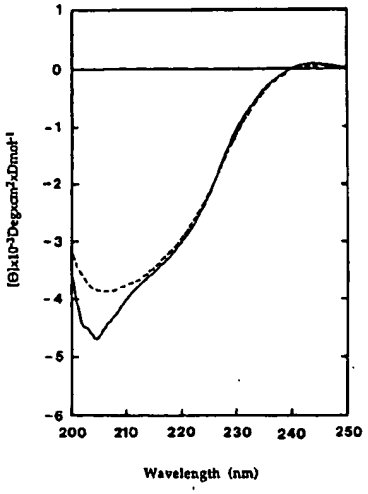

FIG. 5. Far-ultraviolet CD spectra of pure recombinant rat BDNF (-----) and NT-3 (——). See Materials and Methods for details.

cant glycosylation of rat BDNF and NT-3 in Sf-9 cells.

Structural analysis

The far-ultraviolet CD spectra of baculovirus-derived BDNF and NT-3 (Fig. 5) displayed predominant negative band ellipticity at 210 nm in agreement with an earlier report (Radziejewski et al., 1992), although in the present case very little, if any, positive peak at 230 nm was evident. These CD profiles of BDNF and NT-3 are more similar to that reported for native mouse submandibular gland NGF. This difference may be due to the fact that the insect cell recombinant neurotrophins are produced as the homogeneous mature protein, while Radziejewski et al. (1992) used recombinant BDNF and NT-3 that consisted of a mixture of mature and truncated protein species.

Dimerization

In its native conformation, NGF from natural sources (Bothwell and Shooter, 1977) and insect cell–produced human NGF (Barnett et al., 1991) exist as noncovalently associated homodimers. Radziejewski et al. (1992) have shown that recombinant human BDNF and NT-3 also exist as tightly associated dimers at physiologically relevant concentrations. In the latter study, the two recombinant neurotrophins synthesized in Chinese hamster ovary cells occurred as a mixture of the full-length and N-terminal truncated proteins. Under nonreducing conditions and mild sample treatment (no heating in sample buffer containing a low SDS concentration), the dimeric form of mouse (Pettmann et al., 1988) and human (Barnett et al., 1991) NGF is preserved. When purified rat BDNF and NT-3 (reconstituted in 50 m*M* Tris-HCl, pH 8.0) were loaded onto a SDS-PAGE gel, after treatment for 2 min at room temperature with sample buffer that did not contain β-mercaptoethanol, two protein bands corresponding to those of the monomeric and dimeric forms of BDNF or NT-3 were identified (Fig. 6). Densitometry analysis indicated that the relative abundance of the two protein bands in each case was approximately 2:1 in favor of the monomer (data not shown). Human recombinant NGF exhibited a similar behavior (Fig. 6). Boiling the BDNF or NT-3 in SDS- and β-mercaptoethanol–containing sample buffer virtually completely dissociated the dimer into monomers (Fig. 4). When purified native recombinant rat NT-3 was analyzed by gel filtration chromatography, the protein elution volume corresponded to that of ~30 kDa (based on a calibration curve of standard proteins) (Fig. 7), indicating the dimeric nature of the NT-3. Recombinant rat BDNF and human NGF displayed similar elution profiles (data not shown).

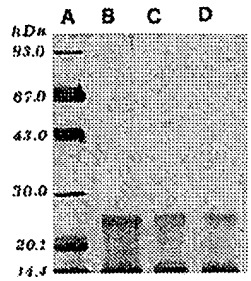

FIG. 6. SDS-PAGE analysis of recombinant rat BDNF and NT-3 under native conditions. Samples (0.4 μg) of the purified protein were run on a 9–22% gradient gel (Phast System) and silver stained. Lane A, molecular mass standards (kDa); lane B, human NGF; lane C, rat NT-3; lane D, rat BDNF.

Conclusion

The results described here indicate that expression of the rat proBDNF and proNT-3 genes in baculo-

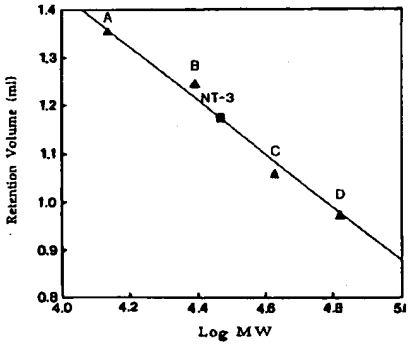

FIG. 7. Gel filtration chromatography of recombinant rat NT-3. Purified NT-3 (5 μg) was applied to a Superdex-75 column (0.3 × 32 cm) with a Smart System (Pharmacia) at 24°C. The elution profile was monitored by absorbance at 280 nm under native conditions. The apparent molecular mass of NT-3 was interpolated from the elution volumes of standard proteins: A, ribonuclease, 13.7 kDa; B, chymotrypsinogen, 25 kDa; C, ovalbumin, 43 kDa; D, bovine serum albumin, 67 kDa. Protein standards (▲) are identified on the graph by their indicated letter symbols. The position of NT-3 is shown (■).

virus-infected insect cells results in a fully folded and properly processed mature BDNF or NT-3 product, respectively. A two-step purification procedure provides purified BDNF and NT-3 with 50% recovery and high specific activity. Furthermore, the present expression system yields quantities of the recombinant proteins that exceed, by at least an order of magnitude, those obtained with other types of eukaryotic cells. Because the BDNF and NT-3 produced in insect cells are homogeneous and lack any N-terminal truncated forms, unlike the case with BDNF synthesized in some mammalian cell expression systems, baculovirus-derived proteins should facilitate recombination experiments between the two factors. As rodent and human BDNF and NT-3 share 100% identity in amino acid sequence, in contrast to the situation for NGF, this source for BDNF and NT-3 provides the quality and quantity of material suitable for further characterizing the properties of BDNF and NT-3, and for eventual clinical application.

REFERENCES

Angeletti R. H. and Bradshaw R. A. (1971) Nerve growth factor from mouse submaxillary gland: amino acid sequence. *Proc. Natl. Acad. Sci. USA* **68**, 2417–2420.

Barde Y.-A., Edgar D., and Thoenen H. (1982) Purification of a new neurotrophic factor from pig brain. *EMBO J.* **1**, 549–553.

Barnett J., Baecker P., Routledge-Ward C., Bursztyn-Pettegrew H., Chow J., Nguyen B., Bach C., Chan H., Tuszynski M. H., Yoshida K., Rubalcava R., and Gage F. H. (1990) Human β nerve growth factor obtained from a baculovirus expression system has potent in vitro and in vivo neurotrophic activity. *Exp. Neurol.* **110**, 11–24.

Barnett J., Chow J., Nguyen B., Eggers D., Osen E., Jarnagin K., Saldou N., Straub K., Gu L., Erdos L., Chaing H.-S., Fausnaugh J., Townsend R. R., Lile J., Collins F., and Chan H. (1991) Physicochemical characterization of recombinant human nerve growth factor produced in insect cells with a baculovirus vector. *J. Neurochem.* **57**, 1052–1061.

Berkemeier L. R., Winslow J. W., Kaplan D. R., Nikolics K., Goeddel D. V., and Rosenthal A. (1991) Neurotrophin-5: a novel neurotophic factor that activates *trk* and *trkB*. *Neuron* **7**, 857–866.

Bothwell M. A. and Shooter E. M. (1977) Dissociation equilibrium constant of β nerve growth factor. *J. Biol. Chem.* **252**, 8532–8536.

Buxser S., Vroegop S., Decker D., Hinzmann J., Poorman R., Thomsen D. R., Stier M., Abraham I., Greenberg B. D., Hatzenbuhler N. T., Shea M., Curry K. A., and Tomich C.-S. C. (1991) Single-step purification and biological activity of human nerve growth factor produced from insect cells. *J. Neurochem.* **56**, 1012–1018.

Davidson D. J., Fraser M. J., and Castellino F. J. (1990) Oligosaccharide processing in the expression of human plasminogen cDNA lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochemistry* **29**, 5584–5590.

Ernfors P., Ibáñez C. F., Ebendal T., Olson L., and Persson H. (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87**, 5454–5458.

Götz R., Kolbeck R., Lottspeich F., and Barde Y.-A. (1992) Production and characterization of recombinant mouse neurotrophin-3. *Eur. J. Biochem.* **204**, 745–749.

Hallböök F., Ibáñez C. F., and Persson H. (1991) Evolutionary studies of the nerve growth factor family reveals a novel member abundantly expressed in *Xenopus* ovary. *Neuron* **6**, 845–858.

Hardy M. R., Townsend R. R., and Lee Y. C. (1988) Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* **170**, 54–62.

Hohn A., Leibrock J., Bailey K., and Barde Y.-A. (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339–341.

Ip N. Y., Ibañez C. F., Nye S. H., McClain J., Jones P. F., Gies D. R., Belluscio L., Le Beau M. M., Espinosa R. III, Squinto S. P., Persson H., and Yancopoulos G. D. (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89**, 3060–3064.

Jarvis D. L. and Summers M. D. (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol.* **9**, 214–223.

Jones K. R. and Reichardt L. F. (1990) Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA* **87**, 8060–8064.

Kaisho Y., Yoshimura K., and Nakahama K. (1990) Cloning and expression of a cDNA encoding a novel human neurotrophic factor. *FEBS Lett.* **266**, 187–191.

Leibrock J., Lottspeich F., Hohn A., Hofer M., Hengerer B., Masiakowski P., Thoenen H., and Barde Y.-A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149–152.

Levi-Montalcini R. (1987) The nerve growth factor thirty five years later. *Science* **237**, 1154–1162.

Luckow V. A. and Summers M. D. (1988) Trends in the development of baculovirus expression vectors. *Biotechnology* **6**, 47–55.

Maisonpierre P. C., Belluscio L., Squinto S., Ip N. Y., Furth M. E., Lindsay R. M., and Yancopoulos G. D. (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* **247**, 1446–1451.

Mobley W. C., Schenker A., and Shooter E. M. (1976) Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry* **15**, 5543–5552.

Mopper K. and Johnson L. (1983) Reverse-phase liquid chromatography analysis of Dns-sugar. *J. Chromatogr.* **256**, 27–38.

Murphy R. A., Chlumecky V., Smillie L. B., Carpenter M., Nattriss M., Anderson J. K., Rhodes J. A., Barker P. A., Siminoski K., Campenot R. B., and Haskins J. (1989) Isolation and characterization of a glycosylated form of β nerve growth factor in mouse submandibular glands. *J. Biol. Chem.* **264**, 12502–12509.

Negro A., Corsa V., Moretto C., Skaper S. D., and Callegaro L. (1992) Synthesis of biologically active brain-derived neurotrophic factor in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **186**, 1553–1559.

Pettmann B., Manthorpe M., Powell J. A., and Varon S. (1988) Biological activities of nerve growth factor bound to nitrocellulose paper by western blotting. *J. Neurosci.* **8**, 3624–3632.

Radziejewski C., Robinson R. C., DiStefano P. S., and Taylor J. W. (1992) Dimeric structure and conformational stability of brain-derived neurotrophic factor and neurotrophin-3. *Biochemistry* **31**, 4431–4436.

Rodriguez-Tebár A. and Barde Y.-A. (1988) Binding characteristics of brain-derived neurotrophic factor to its receptors on neurons from the chick embryo. *J. Neurosci.* **8**, 3337–3342.

Rosenthal A., Goeddel D. V., Nguyen T., Lewis M., Shih A., Laramee G. R., Nikolics K., and Winslow J. W. (1990) Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**, 767–773.

Rosenthal A., Goeddel D. V., Nguyen T., Martin E., Burton L. E., Shih A., Laramee G. R., Wurm F., Mason A., Nikolics K., and Winslow J. W. (1991) Primary structure and biological activity

of human brain-derived neurotrophic factor. *Endocrinology* **129**, 1289–1294.

Saiki R. K., Gelfand D. H., Stoffel S., Scharf S. J., Higuchi R., Horn G. T., Mullis K. B., and Erlich H. A. (1988) Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* **239**, 487–491.

Sambrook J., Fritsch E. F., and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger F., Nicklen S., and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Shaw G. and Kamen E. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediated selective mRNA degradation. *Cell* **46**, 659–667.

Skaper S. D., Facci L., Milani D., Leon A., and Toffano G. (1990) Culture and use of primary and clonal neural cells, in *Methods in Neurosciences, Vol. 2* (Conn P. M., ed), pp. 17–33. Academic Press, San Diego.

Snider W. D. and Johnson E. M. Jr. (1989) Neurotrophic molecules. *Ann. Neurol.* **26**, 489–506.

Summers M. D. and Smith G. E. (1987) A manual of methods for baculovirus vectors and insect cell culture procedure. *Texas Agricultural Experimental Station Bulletin*, No. 1555. Texas A & M University Press, College Station.

Suter U., Angst C., Tien C.-L., Drinkwater C. C., Lindsay R. M., and Shooter E. M. (1992) NGF/BDNF chimeric proteins: analysis of neurotrophin specificity by homolog-scanning mutagenesis. *J. Neurosci.* **12**, 306–318.

Sutter A., Riopelle R. J., Harris-Warrick R. M., and Shooter E. M. (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *J. Biol. Chem.* **254**, 5972–5982.

Thoenen H. (1991) The changing scene of neurotrophic factors. *Trends Neurosci.* **14**, 165–170.

Ullrich A., Gray A., Berman C., and Dull T. J. (1983) Human β-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* **303**, 821–825.